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TITLE: Interaction Between Estrogen Receptor- β and the
Transforming Growth Factor- β Signaling Cascade in Human
Breast Tissue

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13. ABSTRACT (Maximum 200 Words) The concept that interactions between the TGF β and ER signalling pathways occur is not a new one. TGF β has been thought to mediate the anti-proliferative effects of anti-estrogen treatment in breast cancer cells. Apart from direct paracrine and autocrine effects of TGF β on breast cancer cell growth, an increasing amount of evidence suggests that a direct cross-talk between ER and TGF β signalling pathways exists. Here, we report that in Cos1 cells, ER β and ER α inhibit TGF β signalling on the p3TP-lux reporter plasmid in a ligand dependent manner, of which is reversed by anti-estrogen treatment. In MCF7 human breast cancer cells, transient over-expression of ER α inhibits p3TP-lux transcription while anti-estrogen treatment increases activity. Similar results were obtained in a stably over-expressing ER β cell line. We present data to suggest that Ap-1 factors are important in mediating the inhibitory effect of ER on TGF β in Cos1 cells and propose a model for the interaction between ER and TGF β . Our results suggest that a complex interaction between ER, TGF β and Ap-1 may be important in human breast cancer and that changes in any of these signalling pathways during breast tumorigenesis may be involved in altering responses to growth regulatory signals.			
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A. INTRODUCTION

Breast tumorigenesis and breast cancer progression involves the deregulation and hyperactivation of intracellular signalling proteins that lead to uncontrolled cellular proliferation, invasion and eventually, metastasis. During breast cancer development, there is a marked upregulation of estrogen receptor- α (ER α)(1) expression levels that is accompanied by alterations in estrogen responsiveness. Within normal breast epithelium, the majority of proliferating cells are ER α negative(2), while in breast tumors it is the ER α positive cells that are associated with an increase in cellular proliferation and metastasis(3), suggesting that estrogen action changes from that of an indirect mitogen to a direct mitogen. Although the mechanism by which ER becomes deregulated during breast tumorigenesis and breast cancer progression is unknown, the fact that alterations in other factors that enhance ER activity also change during breast cancer development(4) may be an underlying factor. In addition to the ER, expression and cellular responses to the transforming growth factor- β (TGF β) signalling pathway also change during breast tumorigenesis and breast cancer progression. For normal mammary epithelial cells, TGF β is a potent physiological inhibitor of cell cycle progression(5). In breast cancer, however, cells have lost their natural growth inhibitory response to TGF β and may become more aggressive and more likely to metastasize in the presence of this factor(6).

While the expression and activity of ER and TGF β may each be important in the development of breast cancer, alterations in the cross-talk between these two pathways may be equally important. The idea that TGF β may, in part, mediate the anti-proliferative effects of the anti-estrogen tamoxifen has been pursued for some time. In one study, it has been shown that patients receiving tamoxifen therapy have a significant increase in TGF β_1 expression compared to control patients and this induction of TGF β_1 corresponds to an increase in relapse-free survival(7). In contrast, over-expression of TGF β has also been associated with estrogen independence and anti-estrogen resistance in human breast cancer(5). However, apart from the direct autocrine or paracrine effects of TGF β on breast cancer cell growth, direct interactions between the ER and TGF β signalling pathways has been described(8, 9).

B. BODY

In our original research proposal our overall goal was to understand the importance of the interaction between Smad3, a downstream signalling protein of the TGF β signal transduction pathway, and the ER as it pertains to human breast tumorigenesis and breast cancer progression. The specific aims that were to be addressed and the results of each are stated below:

(i) *Define the specificity of the interaction of Smad3 with the ER family, both in vitro and in vivo with co-immunoprecipitations (co-IPs).*

a. **Methods:** co-IPs were performed on either: i) *in vitro* transcribed/translated S³⁵ radiolabelled proteins mixed with appropriate ligands or b) Cos1 cells transiently transfected with ER and Smad3 in the presence of ligands. Proteins were IP with antibodies to ER α , His (recognizes the histidine residues on the tagged ER) or β -galactosidase (negative control). Products were run out on a SDS-Page gel and visualized either by autoradiography or western blot.

b. **Results:** Our results suggest that, in contrast to that previously reported, a direct interaction between ER and Smad3 does not exist. While the discrepancies between our results and that previously published are unclear, differences between cell backgrounds may be influencing the interaction of ER with Smad3. Alternatively, the interaction may be too weak under our conditions to detect.

- (ii) *Define the structural/functional regions of the ER that are required for binding to Smad3.* While the region of ER that specifically binds Smad3 was not previously described, it has been shown by others that Smad1 (another downstream signalling protein of the TGF β pathway) binds to the DNA binding domain of ER(9). Therefore, we did not further pursue this aim.
- (iii) *Determine the structure/function domain of Smad3 that binds to ER.* It has previously been shown by Matsuda et al. that the MH2 domain of Smad3 binds ER(8).
- (iv) *Determine if the interaction between ER and Smad3 affects Smad3 transcriptional activity and, alternatively, if Smad3 affects ER transcriptional activity.*

a. COS1 Cells

- i. Methods: Cos1 cells were transiently transfected with ER (ER α or ER β), Smad3, β -galactosidase reference gene (pCH110) and either an ER responsive luciferase reporter plasmid (vitellogenin ERE and TGF β_3) or a TGF β responsive plasmid (p3TP-lux and collagen 7(A1)-524/+92) in the presence or absence of 10nM estradiol (E $_2$).

ii. Results:

- 1. ERE-II-luc: contains two copies of the vitellogenin A $_2$ estrogen responsive elements upstream of a luciferase gene, representing the classical model of estrogen action (i.e. ER binds directly to DNA). No significant differences in luciferase activity were observed between samples treated with Smad3 and ER compared to those treated with ER alone. This observation is in agreement with that previously reported(10).
- 2. TGF β_3 : contains an estrogen responsive segment from the TGF β_3 promoter upstream of a luciferase reporter gene, representing a non-classical model of estrogen action (i.e. ER does not bind directly to the DNA). No significant differences in luciferase activity was observed between samples treated with Smad3 and ER compared to those treated with ER alone.
- 3. p3TP-lux: contains three TPA-responsive elements of the human collagenase gene upstream of a Smad binding element from the plasminogen activator inhibitor 1 (PAI-1) gene. Cos1 transient transfections with ER and Smad3 suggest that both ER α (p<0.001) and ER β_1 (p<0.05) inhibit p3TP-lux transcription in the presence of estradiol and that this inhibition may be inhibited and subsequently reversed by the presence of the anti-estrogens 4OH-tamoxifen (ER α : p<0.01; ER β_1 : p<0.005) and ICI 182,780 (ER α : p<0.005; ER β_1 : p<0.01). Thus, the effect of ER on Smad3 transcription is ligand dependent. The ER β variants, ER β_2 and ER β_5 , did not affect Smad3 transcription. Addition of E $_2$ and anti-estrogens did not significantly alter protein levels of ER or Smad3 as determined by western blot.
- 4. Collagen 7(A1) -524/+92: contains a segment of the human collagen 7(A1) promoter that consists of a Smad binding element and a consensus Ap-1 site. Initial experiments in Cos1 cells suggested that while activated ER α did not affect collagen transcription (although there was an upward trend), the addition of 4OH-tamoxifen and ICI 182,780 significantly reversed the effect of E $_2$ (p<0.01 and p<0.005, respectively). Similar

results were obtained with ER β ₁. However, upon further studies with this promoter we were unable to obtain an increase in activity upon Smad3 over-expression. As the collagen 7(A1) promoter is strongly activated by Ap-1 factors and since there is a high level of Ap-1 background activity in our Cos1 cells, we are unable to further explore the effects of Smad3 and ER on this promoter.

b. MCF7 Cells

i. Methods: As all of our studies described up to this point were carried out in Cos1 cells (green monkey kidney carcinoma cells), we next wanted to extend our results in the ER positive and TGF β responsive MCF7 human breast cancer cell line. Cells were transiently transfected with Smad3, pRL-tk-luc (a renilla luciferase reference gene) and either p3TP-lux or collagen 7(A1)-524/+92 in the presence or absence of E₂ and the anti-estrogens 4OH-tamoxifen or ICI 182,780.

ii. Results:

1. p3TP-lux: Our results suggest that the addition of E₂ did not have any effect on p3TP-lux activity as compared to vehicle controls. However, as the expression level of ER is relatively low in MCF7 cells (20fmol/mg protein) as compared to that of ER+ human breast tumors (>50fmol/mg protein), we transiently transfected MCF7 cells with ER α , Smad3, pRL-tk-luc and p3TP-lux in the presence or absence of E₂. Results indicate that over-expression of ER α significantly inhibited luciferase activity (p<0.005). This inhibition was not affected by the presence of E₂, although there was a downward trend. However, the addition of 4OH-tamoxifen and ICI 182,780 significantly reversed the inhibition of p3TP-lux by ER α (p<0.0002 and p<0.007, respectively). These results are in agreement with that recently published(11). Western blot and PCR analysis of MCF7 cell extracts suggest that endogenous protein and RNA levels of Smad3 are not altered in the presence of E₂ or anti-estrogens.

Additionally, we also had available to us stably transfected, doxycyclin inducible, ER α and ER β over-expressing MCF7 clones where various levels of ER α or ER β may be achieved by variations in doxycyclin concentration. Transient transfections as described above into ER α over-expressing cells in the presence of doxycyclin were highly variable and thus, did not reach statistical significance. Western blot analysis of protein extracts from these cells show that the level of ER α protein expression widely varies between experiments and the ratio between endogenous ER α and that of the transgene also is altered between samples. Therefore, it may be necessary to categorize these experiments into various levels of ER expression in order to observe a significant change in p3TP-lux transcription. More experiments will need to be performed in order to address this issue. Results with respect to our ER β ₁ over-expressing clones were not as variable as those obtained in the ER α over-expressing cells. 4OH-tamoxifen (p<0.05) and ICI 182,780 (p<0.02) treatment significantly increased p3TP-lux activity in samples treated with doxycyclin as compared to samples treated with vehicle alone. E₂ treatment did not have any effect and western blot analysis of protein

extracts demonstrate that similar levels of transgene was induced between sample replicates.

2. Collagen 7(A1)-524/+92: Results in MCF7 cells transiently transfected with collagen 7(A1), Smad3, pRL-tk-luc and ER suggest that anti-estrogen treatment increases promoter activity ($p < 0.001$) while the presence of over-expressed ER α did not affect luciferase activity. Recently, we obtained a collagen 7(A1) reporter plasmid in which the SBE has been deleted, leaving only the Ap-1 responsive sites. Transient transfection of this reporter plasmid into MCF7 cells demonstrated a similar pattern of activation by anti-estrogens ($p < 0.001$), even though Smad3 over-expression did not affect its activity. Upon comparison of the two reporters, no significant differences in luciferase activity was found, suggesting that the effects of ER is mediated by the Ap-1 binding sites on the collagen 7(A1) promoter and does not involve the SBE in these cells.

Development of a Model System

As described previously, the p3TP-lux reporter plasmid contains three Ap-1 binding sites from the collagenase promoter upstream of the SBE from PAI-1. Evidence suggests that both the SBE and Ap-1 sites are critically important for promoter induction(12) and that these two sites act synergistically(13). In fact, TGF β and the Ap-1 factors, cJun/cFos, have been shown to cooperate on Ap-1 promoters to enhance transcriptional activity that is thought to be the result of an interaction between Smad3 and cJun(13, 14). As ER α has also been suggested to interact with cJun but not with cFos(15, 16), we next wanted to test whether cJun was a limiting factor in the activation of p3TP-lux in the presence of activated ER. Cos1 transient transfections with ER α , Smad3, pCH110, p3TP-lux and cJun or cFos show that over-expression of cJun was able to reverse the inhibition of activated ER α on p3TP-lux activity ($p < 0.05$) while cFos was not. Transfection experiments with ER β_1 suggest that both cJun and cFos significantly ($p < 0.05$ and $p < 0.001$, respectively) reverse the inhibition of p3TP-lux by ER β_1 . As no evidence exists suggesting a physical interaction between ER β_1 and Ap-1 factors, the mechanism through which this reversal occurs is unclear. We hypothesize the following model: in the absence of E $_2$, ER α is unable to interact with cJun and thus, cJun is able to bind to the Ap-1 site of p3TP-lux, activating transcription. In the presence of E $_2$, however, ligand bound ER sequesters cJun away from the promoter thereby, suppressing p3TP-lux activity. Conversely, over-expression of cFos is unable to reverse the inhibition of p3TP-lux in the presence of activated ER α as it does not interact with ER α and is unable to bind DNA without forming heterodimers with Jun(17). To confirm this model, we have attempted several co-IPs between ER and Jun in our Cos1 cells transiently transfected with ER α and cJun in the presence of E $_2$ and TPA (an Ap-1 activator) over various time periods. However, we have been unable to successfully find a direct interaction between ER and Jun *in vivo*. We are currently in the process of testing our hypothesis with electrophoretic mobility shift assays with Cos1 nuclear extracts.

C. KEY RESEARCH ACCOMPLISHMENTS

- Smad3 does not affect ER transcriptional activity on either the vitellogenin ERE or on the TGF β_3 promoter in Cos1 cells.

- ER α and ER β_1 inhibit Smad3 transcriptional activity on the p3TP-lux promoter in Cos1 cells and this inhibition may be inhibited and subsequently reversed by the anti-estrogens 4OH-tamoxifen and ICI 182,780.
- p3TP-lux activity is increased by the anti-estrogens 4OH-tamoxifen and ICI 182,780 in MCF7 cells.
- Collagen 7(A1) activity is increased by anti-estrogen treatment in MCF7 cells and this effect appears to be mediated by the Ap-1 site.
- cJun but not cFos overexpression reverses the effect of activated ER α on p3TP-lux activity in Cos1 cells.
- ER β over-expression in MCF7 cells (doxycyclin inducible) increases p3TP-lux activity in the presence of anti-estrogens while estradiol has no effect.

D. REPORTABLE OUTCOMES

1. Phospho-serine 118 estrogen receptor- α in human breast tumors in vivo. **Cherlet T, Murphy L.** 26th Annual San Antonio Breast Cancer Symposium. San Antonio, TX. 2003. **Appendix 1.**
2. Inhibition of TGF β signaling by estrogen receptors is reversed by cJun but not cFos overexpression. **Cherlet T, Murphy LC.** 94th Annual American Association of Cancer Research. Washington, D.C. 2003. **Appendix 2.**
3. Cross-talk between the transforming growth factor-beta and estrogen receptor signaling pathways. **Cherlet T, Murphy LC.** Era of Hope: Department of Defense Breast Cancer Research Meeting. Orlando, Florida. 2002. **Appendix 3.**
4. Cross-talk between the transforming growth factor beta (TGF-beta) and estrogen receptor (ER) signaling pathways in human breast cancer. **Cherlet T, Murphy LC.** Research Days. Winnipeg, Manitoba. 2002. **Appendix 4.**

E. CONCLUSIONS

Our results suggest that a cross-talk between the ER and TGF β signalling pathways exists. In Cos1 cells, ligand-bound ER α and ER β_1 inhibit TGF β signalling on the p3TP-lux reporter plasmid. As we have been unable to detect a direct interaction between Smad3, a downstream signalling protein of the TGF β signalling pathway and ER in Cos1 cells, we suggest that the mechanism through which ER α inhibits TGF β activity is by sequestering factors (i.e. cJun) away from the p3TP-lux promoter that have previously been shown to be important in activating transcription. The mechanism through which ER β_1 inhibits TGF β signalling in Cos1 cells remains unknown, although Ap1 factors may be involved. In the MCF7 human breast cancer cell line, transient over-expression of ER α inhibits p3TP-lux transcription in the absence of ligand, while anti-estrogen treatment increases TGF β transcriptional activity. Similarly, MCF7 cells stably over-expressing ER β_1 have an increased p3TP-lux promoter activity in the presence of anti-estrogens while E2 has no effect. Therefore, in human breast cancer cells a cross-talk between ER and TGF β exists but appears to be quite complex. Taken together, our results suggest that a complex interaction between ER, TGF β and Ap-1 may be important in human breast cancer and that changes in any of these signalling pathways during breast tumorigenesis and progression may be involved in altering responses to growth regulatory signals seen during these events.

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APPENDIX 1

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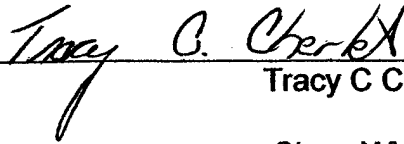
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Tracy C Cherlet, M.Sc. ¹, Adewale Adeyinka Ph.D. ², Yulian Niu ², Linda Snell ², Peter Watson M.D., Ph.D. ² and Leigh Murphy Ph.D. ¹. ¹ Biochemistry and Medical Genetics, University of Manitoba, c/o Cancer Care Manitoba, Winnipeg, Manitoba, Canada, R3E OV9 and ² Pathology, University of Manitoba, Winnipeg, Manitoba, Canada .

Body: Phosphorylation of S-118 of ER α is thought to be important in both the ligand-dependent and ligand-independent activity of ER. Ser¹¹⁸-ER α can be directly phosphorylated by activated MAPK and binding of estradiol to ER *in vitro*. The purpose of this study was to determine if P-Ser¹¹⁸-ER α can be detected in normal and neoplastic breast tissues *in vivo* and to determine its relationship to activated MAPK. H-score analysis and a specific antibody for the IHC detection of P-Ser¹¹⁸-ER α in breast tissue sections were used to compare expression in: (a) human breast tumors and their matched adjacent normal breast tissue and (b) to compare activated MAPK (Erk1/2) to P-Ser¹¹⁸-ER α in breast tumors. P-Ser¹¹⁸-ER α expression was detected in 44.4% of human breast tumors with no detectable staining observed in adjacent matched normal breast tissues. A significant positive association between P-Ser¹¹⁸-ER α expression and ER status was observed while P-Ser¹¹⁸-ER α was found to be inversely related with grade. In addition, a positive correlation of active MAPK and P-Ser¹¹⁸-ER α was found in primary breast tumor sections. These data suggest that P-Ser¹¹⁸-ER α may be a better prognostic factor

and possibly a better biomarker of endocrine treatment responsiveness than total ER.

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A handwritten signature in black ink, appearing to read "Tracy C. Cherlet", is written over a horizontal line.

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INHIBITION OF TGF β SIGNALLING BY ESTROGEN RECEPTORS IS REVERSED BY C-JUN BUT NOT C-FOS OVEREXPRESSION

Tracy Cherlet and Leigh Murphy. University of Manitoba,
Winnipeg, Manitoba, Canada.

Activation of the TGF β signalling cascade has been shown to negatively regulate cellular proliferation. However, many breast cancers are resistant to the growth inhibitory effects of TGF β despite having an apparently intact TGF β pathway. Smad3, a downstream signalling protein of the TGF β pathway, has recently been reported to interact with the estrogen receptor (ER) family thereby modulating ER and TGF β signalling¹. The ER family consists of two members, ER β and ER α . In normal breast tissues, the relative expression of ER β is high while ER α levels are low. During breast tumorigenesis, however, ER β expression decreases while ER α increases dramatically. Therefore, the cross-talk between the TGF β and ER pathways may be important in normal breast tissue that is then altered during human breast tumorigenesis. The goal of this study was to further examine the modulation of TGF β transcriptional activity by ER and the TGF β effects on ER transcription. Cos1 cells were transiently transfected with either the ER reporter plasmids ERE-II-luc (contains two copies of the vitellogenin A₂ estrogen response elements (EREs)) or TGF β ₃-luc or the

Smad3 reporter plasmid p3TP-lux in addition to Smad3, ER and the constitutive β -galactosidase expression vector pCH110. In contrast to previous findings¹, our results suggest that Smad3 does not affect ER α nor ER β transcriptional activity on either the ERE-II-luc or the TGF β ₃-luc promoter. However, ER α and ER β were able to inhibit Smad (p3TP-lux) transcription in a ligand-dependent fashion. This inhibition may be suppressed and subsequently reversed by the anti-estrogens 4OH-tamoxifen and ICI 182,780. The ER β variants, ER β _{2/cx} and ER β ₅, did not affect Smad3 transcription. As both ER α and Smad3 interact with members of the Ap1 family and since the p3TP-lux promoter has three Ap1 binding sites, we next sought to examine whether Ap1 factors may be limiting factors in Smad3 transcription on p3TP-lux. Results suggest that overexpression of c-Jun but not c-Fos was able to reverse the effect of ER α on Smad3 transcription in Cos1 cells. Our results suggest that Ap1 factors may be important in the regulation of TGF β signalling by ER. As ER expression alters during breast tumorigenesis, the cross-talk between the ER and TGF β pathways may be altered and therefore, have an important role in human breast tumorigenesis. (1) Matsuda T, Yamamoto Y, Muraguchi A, Saatcioglu F (2001). J. Biol. Chem. 276(46):42908-42914.

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CROSSTALK BETWEEN THE TRANSFORMING GROWTH FACTOR-BETA AND ESTROGEN RECEPTOR SIGNALING PATHWAYS

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The estrogen receptor (ER) family consists of ER-alpha and ER-beta. In normal breast tissues, expression of ER-beta and its variants is relatively high while ER-alpha levels are low. During breast tumorigenesis, however, ER-beta expression decreases while ER-alpha increases. Therefore, ER-beta may play an important role in normal breast tissues that may be altered throughout breast tumorigenesis. As identification of factors that specifically interact with ER-beta may help to define a putative role for ER-beta, we used the ER-beta2 variant as bait in the yeast two-hybrid screen. Preliminary results suggest that ER-beta interacts with Smad3, a downstream signaling protein of the transforming growth factor-beta (TGF-beta) cascade. Activation of the TGF-beta signaling cascade normally negatively regulates cellular proliferation. However, many breast cancers are resistant to the growth inhibition of TGF-beta despite containing all the components necessary for signal propagation. As Smad3 interacts with other members of the steroid nuclear receptor superfamily, cross-talk between the TGF-beta and ER pathways may exist. We hypothesize that ER-beta and/or its variants interact with the TGF-beta signal transduction pathway and that this interaction modulates TGF-beta signaling. Initially, we examined interactions between ER and Smad3 in vitro. ER and Smad3 were radiolabelled using a coupled transcription/translation system and immunoprecipitated. When low levels of ER-alpha were present, an interaction was observed while at high ER-alpha levels, the interaction was abolished. An interaction between ER-beta1 and Smad3 was also observed. Secondly, we examined whether cross-talk between Smad3 and ER alters either Smad3 or ER transcriptional activity. Cos1 transient transfections with an ERE-CAT suggest that Smad3 does not affect ER-alpha transcription. However, ER-alpha and ER-beta inhibited Smad3 (p3TP-luciferase) transcription in a ligand-dependent fashion. As ER isoform expression and TGF-beta activation altered during breast tumorigenesis, cross-talk between these pathways may have a role in breast tumorigenesis.

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APPENDIX 4

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CROSS-TALK BETWEEN THE TGF- β AND ER SIGNALING PATHWAYS.

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The ER family consists of ER α and ER β . In normal breast tissues, expression of ER β is high while ER α levels are low. During breast tumorigenesis, however, ER β expression decreases while ER α increases. Therefore, ER β may play an important role in normal breast tissues that is altered in breast tumorigenesis. Results from a yeast two-hybrid screen suggest that ER β interacts with Smad3, a signalling protein of the TGF β cascade. Although TGF β normally negatively regulates cellular proliferation, many breast cancers are resistant to TGF β . As Smad3 interacts with other members of the steroid nuclear superfamily, cross-talk between the TGF β and ER pathways may exist. We hypothesize that ER β interacts with the TGF β pathway and that this interaction modulates TGF β signaling. Initially, we examined interactions between ER and Smad3 *in vitro*. ER and Smad3 were radiolabelled using a coupled transcription/translation system and immunoprecipitated. When low levels of ER α were present, an interaction was observed while at high ER α levels, the interaction was abolished. An

interaction between ER β and Smad3 was also observed. Secondly, we examined whether cross-talk between Smad3 and ER alters Smad3 or ER activity. Cos1 transient transfections with an ERE-Luc suggest that Smad3 does not affect ER α nor ER β transcription. However, ER α and ER β inhibited Smad3 (p3TP-Lux) transcription in a ligand-dependent fashion. As ER expression and TGF β activation alter during breast tumorigenesis, cross-talk between these pathways may have a role in breast tumorigenesis.